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Detection and Quantification of Viable but Not Culturable Vibrio Parahaemolyticus in Frozen Bivalve Molluscs

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Abstract : Background: Vibrio parahaemolyticus is a human pathogen that is widely distributed in marine environments. It is frequently isolated from raw seafood, particularly shellfish. Consumption of raw or undercooked seafood contaminated with V. parahaemolyticus may lead to acute gastroenteritis. Vibrio spp. has excellent resistance to low temperatures so it can be found in frozen products for a long time. Recently, the viable but non-culturable state (VBNC) of bacteria has attracted great attention, and more than 85 species of bacteria have been demonstrated to be capable of entering this state. VBNC cells cannot grow in conventional culture medium but are viable and maintain metabolic activity, which may constitute an unrecognized source of food contamination and infection. Also V. parahaemolyticus could exist in VBNC state under nutrient starvation or low-temperature conditions. Aim: The aim of the present study was to optimize methods and investigate V. parahaemolyticus VBNC cells and their presence in frozen bivalve molluscs, regularly marketed. Materials and Methods: propidium monoazide (PMA) was integrated with real-time polymerase chain reaction (qPCR) targeting the tl gene to detect and quantify V. parahaemolyticus in the VBNC state. PMA-qPCR resulted highly specific to V. parahaemolyticus with a limit of detection (LOD) of 10-1 log CFU/mL in pure bacterial culture. A standard curve for V. parahaemolyticus cell concentrations was established with the correlation coefficient of 0.9999 at the linear range of 1.0 to 8.0 log CFU/mL. A total of 77 samples of frozen bivalve molluscs (35 mussels; 42 clams) were subsequently subjected to the qualitative (on alkaline phosphate buffer solution) and quantitative research of V. parahaemolyticus on thiosulfate-citrate-bile salts-sucrose (TCBS) agar (DIFCO) NaCl 2.5%, and incubation at 30°C for 24-48 hours. Real-time PCR was conducted on homogenate samples, in duplicate, with and without propidium monoazide (PMA) dye, and exposed for 45 min under halogen lights (650 W). Total DNA was extracted from cell suspension in homogenate samples according to bolliture protocol. The Real-time PCR was conducted with species-specific primers for V. parahaemolitycus. The RT-PCR was performed in a final volume of 20 µL, containing 10 µL of SYBR Green Mixture (Applied Biosystems), 2 µL of template DNA, 2 µL of each primer (final concentration 0.6 mM), and H2O 4 µL. The qPCR was carried out on CFX96 TouchTM (Bio-Rad, USA). Results: All samples were negative both to the quantitative and qualitative detection of V. parahaemolyticus by the classical culturing technique. The PMA-qPCR let us individuating VBNC V. parahaemolyticus in the 20,78% of the samples evaluated with a value between the Log 10-1 and Log 10-3 CFU/g. Only clams samples were positive for PMA-qPCR detection. Conclusion: The present research is the first evaluating PMA-qPCR assay for detection of VBNC V. parahaemolyticus in bivalve molluscs samples, and the used method was applicable to the rapid control of marketed bivalve molluscs. We strongly recommend to use of PMA-qPCR in order to identify VBNC forms, undetectable by the classic microbiological methods. A precise knowledge of the V.parahaemolyticus in a VBNC form is fundamental for the correct risk assessment not only in bivalve molluscs but also in other seafood.

Keywords : food safety, frozen bivalve molluscs, PMA dye, Real-time PCR, VBNC state, Vibrio parahaemolyticus
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